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Unusual Levels of Heat Shock Element-Binding Activity in Embryonal Carcinoma Cells

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In contrast to differentiated somatic cells, mouse embryonal carcinoma (EC) cell lines spontaneously express high levels of major members of the heat shock protein (HSP) family. In addition, some EC cell lines (noninducible) are not able to induce HSP gene transcription and HSP synthesis after a stress. However, after in vitro differentiation, constitutive HSP expression decreases and the differentiated derivatives become able to induce HSP gene transcription after a stress. These cells were tested by gel shift assays for the presence of an activity able to bind the heat shock element (HSE) before and after a stress. Control fibroblasts grown at 37°C did not contain significant levels of HSE-binding activity, but heat shock dramatically increased the level of HSE-binding activity. In contrast to control fibroblasts, all EC cells contained significant levels of HSE-binding activity at 37°C. In the inducible EC cell line F9, as in fibroblasts, heat shock strongly increased the level of HSE-binding activity. In the noninducible EC cells, however, HSE-binding activity markedly decreased upon heat shock. During in vitro differentiation of the noninducible cell line PCC7-S-1009, the constitutive HSE-binding activity found at 37°C disappeared and heat induction of the HSE-binding activity appeared. Therefore, a good correlation exists between the high spontaneous expression of some members of the HSP family and the constitutive level of HSE-binding activity in EC cells at 37°C. Heat induction of HSP gene transcription correlates with a strong increase in HSE-binding activity, whereas a deficiency in heat induction of HSP gene transcription is associated with a loss of HSE-binding activity upon heat shock.

The heat shock response provides a model system for the study of gene regulation in eucaryotes (reviewed in references 8 and 27). Indeed, in almost all cells, induction of the heat shock proteins (HSPs) upon exposure to a wide range of stresses is the result of a dramatic increase in the transcription of heat shock genes.

A short, highly conserved sequence, the heat shock element (HSE), is required for transcriptional activation of heat shock genes (13, 19, 34, 39). This HSE, identified by deletion mapping and site-specific mutagenesis, is able to confer stress inducibility when fused to a heterologous gene in insect or mammalian cells or in injected *Xenopus* oocytes (10, 35). HSEs are binding sites for a specific transcription factor, the heat shock factor (HSF). HSF, originally purified from *Drosophila* Kc cells, is required for *Drosophila* HSP70 gene transcription both in vitro (33, 44, 46) and in vivo (48). An HSE-binding activity has also been identified in yeast cells (46, 40) and in human HeLa cells (23, 31). The yeast HSF seems to be encoded by a single gene, which has been recently cloned (41, 45).

Induction of heat shock genes is not blocked by inhibitors of protein synthesis, which suggests that HSF already exists in the cell in an inactive form at normal temperatures. Therefore, the crucial step for heat activation of heat shock genes seems to be the conversion of inactive HSF into an active form.

In human and *Drosophila* cells, the inactive form of HSF is not able to efficiently bind HSE but acquires strong HSE-binding activity during its conversion to an active form (23, 40, 51). The posttranslational modifications involved in this conversion have not been yet identified. In human cells, HSF activation seems to be a two-step process. One step, the acquisition of DNA-binding activity, could be obtained in vitro by heating human control cell extracts at temperatures known to provoke HSF activation in vivo (25). In contrast to what happens in *Drosophila* and human cells, heat shock does not modify the HSE-binding activity of yeast HSF, which is able to bind HSE at all temperatures both in vitro (40, 41) and in vivo (22). Therefore, in this species, the regulation of HSF activity does not occur at the DNA-binding step but involves the ability to activate transcription. The ability of the bound HSF to promote transcription is modulated by growth temperature and is correlated with the extent of phosphorylation of the polypeptide (41).

Embryonal carcinoma (EC) cells and early mouse embryonic cells not only have common biochemical, immunological, and differentiation properties (9, 43) but also display very similar patterns of HSP expression (5, 30). They spontaneously express HSP86 and HSP84 (also termed HSP89s and HSP89f) and HSC73 (referred to as HSP70 in our previous reports) at high levels (3, 5, 30). However, induction of HSP68 synthesis cannot be obtained after stress either in PCC4-AzaR1 (PCC4) or PCC7-S-1009 (1009) EC cell lines (30, 47). We had previously shown that transcription of the corresponding HSP68 gene (also termed HSP72 and encoding a strictly inducible protein) is not induced in these cells. Moreover, transcription driven by an exogenous promoter, the *Drosophila* HSP70 gene promoter, also cannot be heat induced (29). After in vitro differentiation of the noninducible cell line 1009, transcription of these endogenous and exogenous heat shock genes becomes strongly heat inducible. One explanation may be that noninducible EC cells fail to produce an active form of HSF in response to heat shock. To address this question, we used gel shift assays to test various fibroblasts and EC cell lines for the presence of HSE-binding activity before and after a heat shock.

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We found that, in contrast to fibroblasts, EC cells contained significant levels of HSE-binding activity at normal temperatures. In the inducible EC cell line F9, heat shock strongly increased the level of HSE-binding activity, as it did in fibroblasts. In the noninducible EC cell lines PCC4 and 1009, however, HSE-binding activity was lost upon heat shock. In vitro differentiation of the noninducible cell line 1009 resulted in a decrease in the constitutive HSE-binding activity found at 37°C and the appearance of strong induction of HSE-binding activity upon heat shock. Therefore, a good correlation exists between the high spontaneous expression of some members of the HSP family and the constitutive level of HSE-binding activity in EC cells at 37°C. Heat induction of HSP gene transcription correlates with a strong increase in HSE-binding activity, whereas deficiency in heat induction of HSP gene transcription is associated with a loss of HSE-binding activity upon heat shock.

MATERIALS AND METHODS

Cell culture and stress conditions. EC cells were grown at 37°C in tissue culture flasks with Dulbecco modified Eagle medium supplemented with 15% fetal calf serum in a 12% CO_2 atmosphere. F9 cells were plated on gelatin-coated tissue culture flasks. F9 and PCC4-AzaR1 are derived from the same teratocarcinoma OTT 6050 cell line (obtained by transplantation of a 6.5-day-old embryo). PCC7-S-1009 is derived from a spontaneous teratocarcinoma in testes of 129 × C57BL/6J mice. 3-TDM1 (TDM) is a trophectodermal cell line established from in vivo transfer of PCC3 tumors and cloned in culture (the PCC3 cell line is a derivative of OTT 6050). TDM was grown in the same manner as EC cell lines (for reviews of these cell lines, see references 32 and 43). For in vitro differentiation experiments, retinoic acid (Sigma Chemical Co.) was added at 0.2 μM (final concentration), and 1 day later 1 mM dibutyryl-cAMP was added for 3 more days, with a medium change every 48 h (36). 3T6 or L tk^- $aprt^-$ fibroblasts were grown with 10% fetal calf serum. For heat shock, culture flasks were immersed in a 45°C water bath for 15 min.

Plasmids. Plasmid pM1.8, provided by R. Morimoto, recognizes the specifically heat-inducible mRNAs coding for HSP68 (50). Plasmid pAL41 was provided by S. Alonso. It contains a 1.15-kilobase-pair kb fragment of murine B nonmuscle actin cDNA cloned between two *Pst*I sites (1). Plasmid pG19, provided by V. Legagneux, contains a 700-base-pair *Hin*dIII-*Hin*dIII fragment encoding part of the 3′ region of the murine HSP86-coding sequence (V. Legagneux et al., Differentiation, in press).

Plasmid pPB1 was provided by Jacques Piette. It contains the *Pvu*II-*Pvu*II fragment 4 of the polyomavirus regulatory region inserted between the *Eco*RI and *Sal*I sites of pML2 (37). The *Pvu*II-*Pvu*II fragment contains the polyomavirus B enhancer.

Run-on experiments. Run-on experiments were performed as previously described (12). Filters for the slot blot were prepared with a Schleicher & Schuell device. Each filter was loaded with 2 μg of the *Pst*I-*Pst*I fragment of pAL41 corresponding to the actin fragment, 2 μg of the *Sac*II-*Bam*HI fragment of plasmid pM1.8 corresponding to the coding part of the HSP68 gene insert, and, in the experiment using PCC4 nuclei, 2 μg of the *Hin*dIII-*Hin*dIII fragment of the HSP86 cDNA fragment of plasmid pG19. Densitometer scanning of the autoradiograms provided an estimate of the induction of HSP gene transcription. The values reported were normalized to those for actin gene transcription.

Preparation of whole-cell extracts. Subconfluent culture flasks (about 4×10^7 cells) were gently agitated at 37°C for 30 min before extraction or before heat shock. Extraction was performed essentially as described by Zimarino and Wu (51).

Cells were rapidly rinsed with phosphate-buffered saline and trypsinized in 10 ml. Cells were immediately transferred at 4°C, and 4 ml of fetal calf serum in 10 ml of phosphate-buffered saline was added to stop trypsinization. Cells were rapidly pelleted and immediately frozen in liquid N_2.

Whole-cell extracts were prepared from frozen pellets by thawing and pipetting in 5 volumes of extraction solution [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.4 M NaCl, 0.1 mM ethylene glycol-bis(β-aminoethyl ether) (EGTA), 0.5 mM dithiothreitol, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride] until the suspension was visually homogeneous. The time of extraction did not exceed 10 min. The extracts were clarified by centrifugation at 100,000 × *g* for 5 min, and the supernatant (4 to 9 mg/ml of protein) was stored in liquid N_2.

Analysis of the mobility of DNA-protein complexes by gel electrophoresis. Oligonucleotides were synthesized by a Gene Assembler (Pharmacia, Inc.). One strand was ^{32}P end labeled before annealing, using T4 polynucleotide kinase and [γ-^{32}P]ATP.

Binding reactions were typically performed in a final volume of 23 μl. Various amounts of extracts made up to 14 μl with extraction solution were mixed with 9 μl of binding solution containing 0.2 ng of the ^{32}P-labeled double-stranded oligonucleotide, 4 μg of double-stranded poly(dI-dC), 9% (wt/vol) Ficoll, 44 mM HEPES (pH 7.6), 2.2 mM $MgCl_2$, and 88 mM KCl. Final concentrations were 26 mM HEPES (pH 7.6), 0.24 M NaCl, 40 mM KCl, 1 mM $MgCl_2$, 0.3 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 0.06 mM EGTA, 4% Ficoll, and 3% glycerol. Final concentrations of NaCl ranging from 0.14 to 0.24 M did not lead to detectable modifications of binding characteristics.

After 10 min at room temperature, the reaction mixtures were loaded on a 4% acrylamide gel (acrylamide/bisacrylamide ratio of 30:1) in 0.5× TBE (44.5 mM Tris [pH 8.0], 1 mM EDTA, 44.5 mM boric acid). Gels were preelectrophoresed for 2 h at 100 V (12 to 20 mA), and buffer was recirculated during both prerunning and sample electrophoresis. Dried gels were autoradiographed at −70°C with Kodak X-OMAT film and a Dupont Cronex Lightning-Plus intensifying screen.

Densitometric scanning of the autoradiograms provided an estimate of the relative amounts of specific DNA-protein complexes. The mean values of three independent experiments are given in Table 1. These values were determined from autoradiograms obtained after various exposure times.

In experiments involving the B enhancer of polyomavirus, plasmid pPB1 was digested by *Eco*RI and *Sal*I, and the fragment containing the B enhancer of polyomavirus was purified, labeled by nick translation, and used for gel shift experiments in the presence of various cell extracts.

RESULTS

Heat inducibility of HSE-binding activity in inducible mouse cell lines. (i) Properties of the HSE-binding activity of murine fibroblasts. To detect activities that would bind to the HSE in mouse cells, whole-cell extracts were mixed with labeled HSE containing oligonucleotide fragments and analyzed by gel shift assays. We used an oligonucleotide containing two overlapping HSEs (HSE2; Fig. 1). These sites perfectly match the previously defined HSE consensus sequence

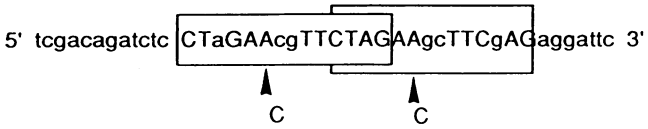

FIG. 1. Nucleotide sequences of HSE2 and HSE2C oligonucleotides. Nucleotides that perfectly match the consensus sequence defined by Pelham and Bienz (35) are in capital letters. Symbols: ▭, extent of one single HSE; ▲, single mutation introduced in each HSE of HSE2C.

(CNNGAANNTTCNNG) (35). Overlapping HSEs appear to be strong binding sites for HSF and frequently occur in heat shock gene promoters of numerous organisms (for a review, see reference 8).

To test the specificity of the interactions of the detected binding activities with HSE2, we used the oligonucleotide HSE2C, which contains a single mutation in both HSEs. Recently, Amin et al. (2) showed that a functional HSE includes a minimum of three GAA segments at 2-nucleotide intervals and in alternating orientations. This requirement was satisfied in HSE2. Amin et al. (2) observed that replacement of GAA by GAC in the third GAA block of a *Drosophila* regulatory element resulted in a 10-fold inactivation of the promoter. Therefore, we chose to replace GAA by GAC in the first GAA block of the two HSE consensus sequences in oligonucleotide HSE2 (compare HSE2 and HSE2C in Fig. 1).

We chose to routinely measure HSE-binding activity in whole-cell extracts because this procedure avoids loss of material during subcellular fractionation, as occurs in nuclear extract preparations, and because the rapidity of preparation of whole-cell extracts reduces protein degradation and the risk of alteration of binding activity. However, experiments with nuclear extracts (prepared as described by either Dignam et al. [11] or Parker and Topol [33]) gave comparable results (data not shown). We performed our assays with an excess of DNA probes so that the intensity of specific complexes would be a fair estimate of the relative HSE-binding activity in extracts with equivalent numbers of cells (or equivalent amounts of proteins).

TABLE 1. Comparison of amounts of HSE2 found in specific complexes before and after heat shock in extracts of different cell lines

Cell line	Complexed HSE2 (arbitrary units)	
	Per 2×10^5 cells	For equal amounts of total proteins
3T6		
Control	1	1
Heat shocked	>150	>172
F9		
Control	19	24
Heat shocked	180	250
PCC4		
Control	10	33
Heat shocked	1	4
1009		
Control	21	32
Heat shocked	4	8

Very little or no HSE-binding activity was detected in extracts from control 3T6 fibroblasts grown at 37°C (Fig. 2A, lane 2). After a 15-min heat shock, HSE-binding activity was strongly induced: the amount of HSE2 found complexed with proteins increased by more than 150-fold (Fig. 2A, lane 3; Table 1). Similar results were obtained with another fibroblastic cell line, L tk^- $aprt^-$ (data not shown). The binding activity detected in heat shock fibroblast extracts resolved into two major complexes (Fig. 2B). This activity was highly specific of HSE: an approximately threefold excess of unlabeled HSE2 was sufficient to abolish 50% of the signal (Fig. 2B, lanes 2 to 7). Oligonucleotide HSE2C did not compete efficiently with HSE2. A decrease of 50% of the signal required about 10-fold more HSE2C than HSE2 (Fig. 2B, lanes 8 to 13). From these results, we conclude that heat shock strongly induced an activity that specifically bound to HSE in mouse fibroblastic cells.

(ii) Properties of HSE-binding activities of the inducible EC cell line F9. In the EC cell line F9, transcription of the HSP86 (Legagneux et al., in press) and HSP68 (Fig. 3A) heat shock genes is strongly heat induced (25- and 10-fold, respectively,

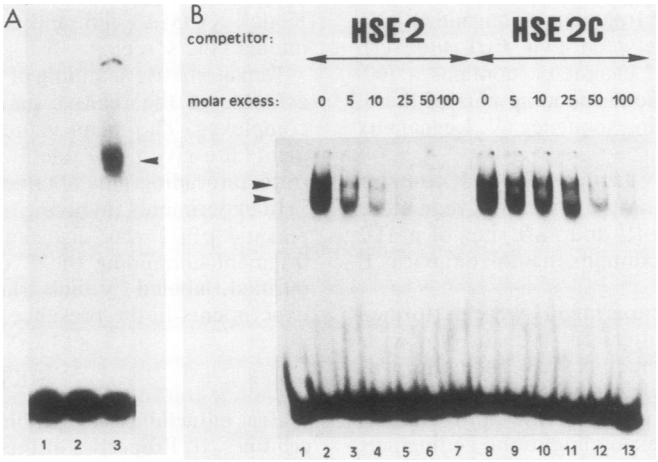

FIG. 2. (A) Gel electrophoresis of HSE-protein complexes in 3T6 control (C; lane 2) and heat-shocked (HS; lane 3) cell extracts. Cell extracts (equivalent to 2×10^5 cells) were mixed with labeled HSE2 except in lane 1 (0), to which no extract was added and on which only unbound DNA is seen. Arrowheads mark specific complexes (which did not resolve into two forms in this experiment). (B) Competitive inhibition between HSE-protein complexes and unlabeled HSE2 (lanes 2 to 7) or HSE2C (lanes 8 to 13). Heat-shocked 3T6 cell extract (equivalent to 2×10^5 cells) was mixed with 0.2 ng of labeled HSE2 and the amounts of competitor as indicated. No extract was added in lane 1.

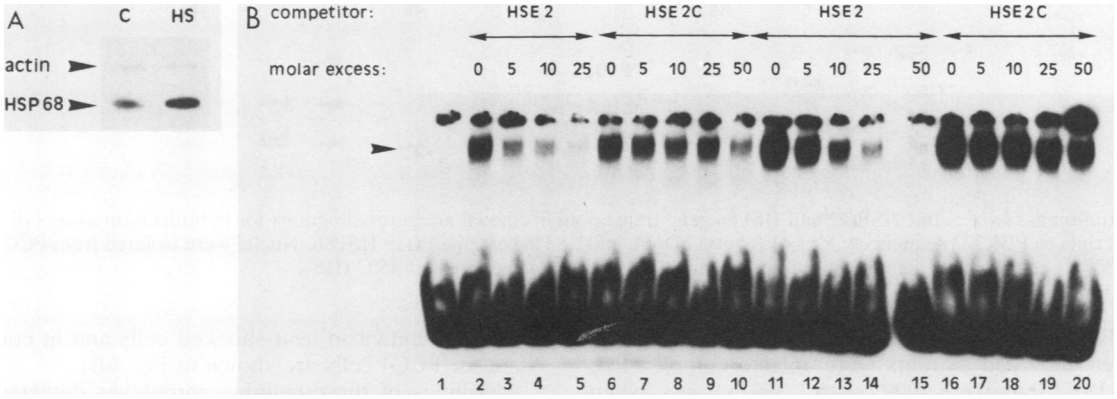

FIG. 3. (A) Run-on assay of actin and HSP68 gene transcription in F9 cells. Autoradiograms are of the hybridization of ^{32}P-labeled run-on transcripts with pAL41 β-actin cDNA and pM1.8 HSP68 probes. Nuclei were isolated from F9 cells without heat shock (C) or 30 min after a 15-min heat shock at 45°C (HS). (B) Competitive inhibition between HSE-protein complexes and unlabeled HSE2 (lanes 3 to 5 and 12 to 15) and HSE2C (lanes 7 to 10 and 17 to 20). F9 control (lanes 2 to 10) and heat-shocked (lanes 11 to 20) cell extracts (equivalent to 2×10^5 cells) were mixed with 0.2 ng of labeled HSE2 and the amounts of competitor indicated. No extract was added in lane 1. ►, Positions of specific complexes.

when normalized to values for actin gene transcription). We investigated whether an HSE-binding activity was also heat induced in this cell line.

In contrast to fibroblasts, F9 control cell extracts contained significant level of HSE-binding activity (Fig. 3B, lane 2; Table 1). However, as in fibroblasts, heat shock resulted in the induction of HSE-binding activity (Fig. 3B, compare lanes 2 and 6 with lanes 11 and 16; Table 1). The amount of complexed HSE2 was equal in 3T6 and F9 heat-shocked cells when extracts prepared from equivalent numbers of cells were compared (Table 1). Extended gel runs also resolved the binding activities detected in F9 control or heat-shocked cells into two major complexes (Fig. 4, lane 3). No differences between F9 control and heat-shocked cells in mobility or in ratio of the two major complexes could be detected (data not shown). Complexes detected in extracts of both control and heat-shocked cells were formed by specific interactions; 50% competition was obtained with an approximately threefold excess of unlabeled HSE2 in both control and heat-shocked cell extracts, whereas HSE2C did not compete efficiently (Fig. 3B). From these results, we conclude that in F9 cells as in fibroblasts, heat shock increased the level of HSE-specific binding activity. Both cell lines gave rise to two major complexes. The complexes detected with F9 heat-shocked cells displayed a slightly slower mobility than did those of 3T6 heat-shocked cells (Fig. 4) but were similarly displaced by competitors (Fig. 2B and 3B). Thus, in fibroblastic and embryonic cell lines in which HSP gene transcription was strongly heat inducible, high and comparable levels of HSE-binding activity were rapidly induced by heat shock.

High constitutive levels of HSE-binding activity in EC cell lines. Run-on experiments showed that transcription of HSP86 and HSP68 genes was not heat inducible in PCC4 and 1009 EC cell lines or in the trophectodermal cell line TDM (Fig. 5; data for HSP86 are shown only for PCC4 cells; the decrease in actin gene transcription was related to the decrease in general transcription). Moreover, an exogenous heat shock promoter was not able to drive expression of the chloramphenicol acetyltransferase (CAT) gene in such heat-treated EC cell lines (29). Therefore, we tested cell lines that display this phenotype for the presence of HSE-binding activity and its activation after a stress: two EC cell lines, PCC4 and 1009, and the trophectodermal cell line TDM.

Similar to the EC cell line F9, noninducible EC cells (Fig. 6A and C; Table 1) and TDM (data not shown) contained significant levels of HSE-binding activity in the absence of stress. For instance, 1009 and F9 control cell extracts were able to bind equal amounts of HSE2; these extracts bound 20 to 25 times more HSE2 than did 3T6 control cells (when equivalent numbers of cells were compared) (Fig. 6A; Table 1). The complexes detected in noninducible EC and TDM cells were specific for HSE, since an approximately threefold excess of unlabeled HSE2 was sufficient to abolish 50% of the signal. Moreover, HSE2C did not efficiently compete with HSE2 (Fig. 6C and data not shown).

Decrease in HSE-binding activity upon heat shock in noninducible EC cells. In contrast to the results for inducible cells, heat shock did not induce HSE-binding activity in noninducible EC cells; HSE-binding activity was found in

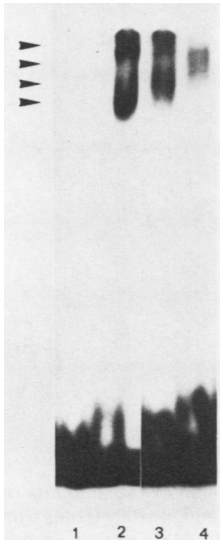

FIG. 4. Analysis of the mobilities of HSE-protein complexes by long-gel-run electrophoresis. Labeled HSE2 probe was loaded alone (lane 1) or with control (lane 4) or heat shocked (lane 2 and 3) cell extracts (equivalent to 2×10^5 cells) prepared from 3T6 (lane 2), F9 (lane 3), or PCC4 (lane 4) cells. , Positions of specific complexes. Different exposure times were used for lanes 2 and 3.

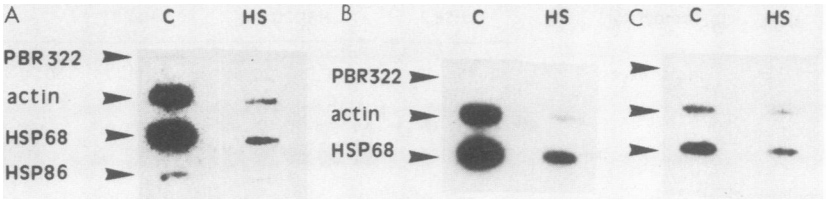

FIG. 5. Run-on assay of actin, HSP68, and HSP86 gene transcription. Shown are autoradiograms for hybridization assays of ^{32}P-labeled run-on transcripts to PBR322 sequences. PAL41 β-actin cDNA, pM1.8 HSP68, and pG19 HSP86. Nuclei were isolated from PCC4 (A), 1009 (B), and TDM (C) cells without heat shock (C) or 30 min after a 15-min heat shock at 45°C (HS).

lower levels than in control cells (Fig. 6A). For instance, heat-shocked 1009 cell extracts were able to complex five times less HSE2 than were 1009 control cell extracts (when 2×10^5 cells were considered) (Table 1). This decrease was specific for HSE-binding activity, since the factor recognized by the polyomavirus B enhancer was found to be equally abundant in heat-shocked cells and in control cells (data for PCC4 cells are shown in Fig. 6B).

Mobilities of the remaining complexes detected in heat-shocked cell extracts were identical to those in control cells. Extended gel runs showed that complexes derived from the different cell lines exhibited slightly different mobilities (Fig.

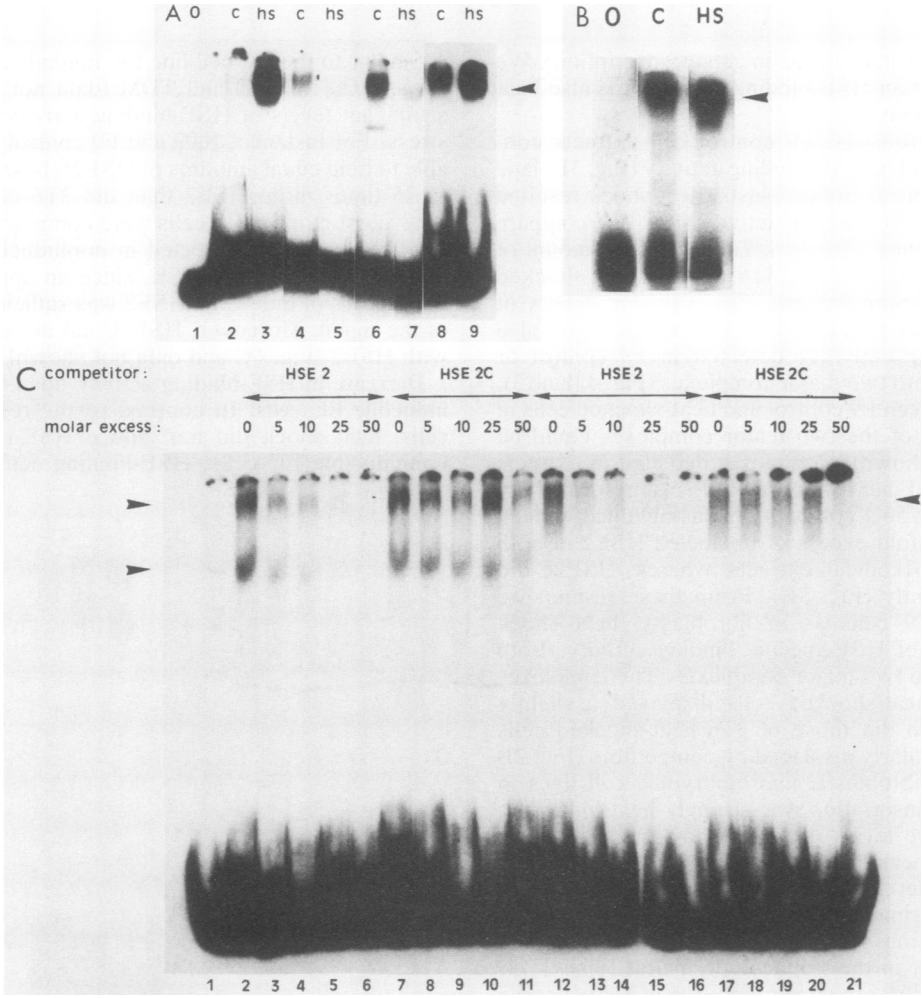

FIG. 6. (A) Comparison of the amounts of HSE-binding activity present in control (c) or heat-shocked (hs) cells of cell lines 3T6 (lanes 2 and 3), PCC4 (lanes 4 and 5), 1009 (lanes 6 and 7), and F9 (lanes 8 and 9). Extracts equivalent to 2×10^5 cells were mixed with labeled HSE2. ◄ , Positions of specific HSE-protein complexes. In lane 1, no extract was added (giving the position of unbound HSE2). (B) Comparison of the amount of specific factor bound to the polyomavirus B enhancer in PCC4 cells before and after heat shock. Extracts from 3×10^5 cells were incubated with the labeled *Eco*RI-*Sal*I fragment of plasmid pPB1. Lanes: 0, no extract added (giving the position of the unbound DNA fragment); C, control cell extract; HS, heat-shocked cell extract. (C) Competitive inhibition of HSE-protein complexes by unlabeled HSE2 (lanes 3 to 6 and 13 to 16) and unlabeled sequence HSE2C (lanes 8 to 11 and 18 to 21). Lane 1, Unbound labeled HSE2 probe. Control cell extract (equivalent to 2×10^5 cells) of 1009 (lanes 2 to 11) or PCC4 (lanes 12 to 21) cells was mixed with labeled HSE2 and the amounts of competitor is indicated.

4). These variations were not simply associated with the inducibility or noninducibility of the cell lines. The 3T6, F9, 1009, and TDM cell lines gave rise to two major forms of complexes (Fig. 2B, 4, and 6C), whereas only one complex was detected with PCC4 cells (Fig. 4 and 6C). These two forms may have resulted from differential occupation of the two sites of HSE2 or from multimeric association of HSE-binding activity (for analogy with another system, see reference 38). The variations in mobilities observed between the different cell lines may have resulted from different post-translational modifications of the HSE-binding activity, such as phosphorylation or glycosylation (21).

Finally, these results show that in all cell lines tested, deficient activation of heat shock gene transcription correlated with deficient activation of HSE-binding activity by stress.

Decrease in constitutive level and heat-induced activation of HSE-binding activity during 1009 cell differentiation. As previously mentioned, the exogenous *Drosophila* HSP70 gene promoter was not able to stimulate expression of the CAT gene upon heat shock in 1009 cells. However, after differentiation of the 1009 cell line, this promoter was able to direct heat-induced expression of the CAT gene (29). Therefore, we asked whether heat induction of an HSE-binding activity would appear upon differentiation of the 1009 cell line. Cells that had been treated for 1 day with retinoic acid and dibutyryl-cAMP displayed the same characteristics as did undifferentiated 1009 cells: control cell extracts contained high levels of HSE-binding activity, whereas in heat-shocked cell extracts HSE-binding activity was not induced but, on the contrary, found at much lower levels (by about fivefold) than in control cells (Fig. 7). After 3 days of treatment, however, 1009 cells showed a pattern similar to that of fibroblastic cells in gel shift assays. The level of HSE-binding activity in control cells was considerably lower (almost undetectable), and heat shock strongly induced HSE-binding activity (Fig. 7). The complexes detected with control or heat-shocked cell extracts showed the same mobilities before and after differentiation and the same characteristics in competition experiments (data not shown). Therefore, the appearance of efficient heat stimulation of transcription via a heat shock promoter during 1009 cell differentiation correlated with the appearance of efficient heat activation of HSE-binding activity, and the decrease in constitutive HSP gene expression correlated with a decrease in the amount of HSE-binding activity in control cells.

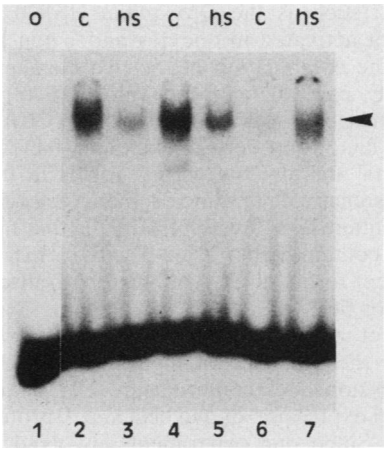

FIG. 7. Analysis by gel electrophoresis of HSE-protein complexes in whole-cell extracts of undifferentiated 1009 cells (lanes 2 and 3) and derivatives after differentiation treatment for 1 (lanes 4 and 5) or 3 (lanes 6 and 7) days. Labeled HSE2 oligonucleotide was mixed with either no extract (0, lane 1; showing the position of unbound HSE2), 40 μg of control cell extracts (c; lanes 2, 4, and 6), or 40 μg of heat-shocked cell extracts (hs; lanes 3, 5, and 7). ◄, Positions of specific HSE-protein complexes. These results allow a good estimate of the level of induction of HSE-binding activity for a given differentiated state but do not allow a comparison of absolute levels of HSE-binding activity among cells at different stages of differentiation.

DISCUSSION

We have previously shown that some EC cell lines are deficient in transactivation of heat shock genes. We therefore hypothesized that these cells may lack a positive transcription factor that would appear during in vitro differentiation (29). In this study, we investigated the presence of HSE-binding activity in both heat-inducible cells (fibroblasts and EC F9) and noninducible cell lines.

Murine fibroblasts stimulated by heat shock contained high levels of HSE-binding activity, whereas nonshocked cells had only low basal level of activity. Thus, in murine fibroblasts, as in *Drosophila* culture cells but in contrast to yeast cells, a crucial step for the activation of heat shock genes may be the modification of HSF that allows binding on a heat shock promoter.

With regard to fibroblasts, we asked whether heat shock induced HSE-binding activity in noninducible EC cell lines. We made the two following observations. First, all non-shocked EC cells spontaneously contained significant levels of HSE-binding activity. Second, noninducible EC cell lines were not able to induce HSE-binding activity upon heat shock; on the contrary, they contained strongly reduced levels of HSE-binding activity after a heat shock.

Involvement of constitutive HSE-binding activity in high spontaneous expression of HSP86 and HSC73 in EC cells. EC cells overexpress two major heat shock proteins, HSC73 and HSP86 (5, 30). This is due (partially, in the case of HSC73) to an increased rate of transcription relative to that of differentiated derivatives or fibroblasts (16; Legagneux et al., in press).

Our results support the hypothesis that HSF may be involved in basal HSP gene transcription. Indeed, a good correlation exists between the spontaneous abundance of HSE-binding activity and expression of HSP86 and HSC73 in EC cells and their differentiated derivatives (Fig. 6A and 7). First, all EC cells lines contain high levels of HSE-binding activity; and second, this activity decreases upon differentiation of 1009 cells (Fig. 7) and F9 cells (data not shown) to reach levels comparable to those found in fibroblasts, as do the abundance of HSP86 and HSC73 proteins and transcription of the encoding genes.

The involvement of HSF in HSP gene expression independently of heat induction has been suggested for other systems (for a review, see reference 8). It has been suggested that binding of auxiliary factors to regulatory elements distinct from HSE may favor binding of the inactive form of HSF (or of rare molecules of active HSF) to HSE and thus allow transcription of heat shock genes (8). For instance, *Xenopus* HSP70 gene expression in oocytes provides indirect evidence for the involvement of HSF in combination with an auxiliary factor; even though *Xenopus* HSP70 genes are strictly inducible in transfected somatic cells, they are constitutively expressed in injected oocytes (6). This constitutive expression requires the proximal HSE but also the proximal CCAAT sequence (7). Genes that do not contain a

CCAAT box, such as the *Drosophila* HSP70 and HSP30 genes, are not activated in oocytes under nonshock conditions. Only the combination of the two elements, CCAAT box and HSE, can efficiently activate HSP70 genes under nonshock conditions, which suggests that a CCAAT-binding transcription factor may act as an auxiliary factor. However, both elements are also necessary for HSP70 gene heat induction in somatic cells. Since somatic cells do not spontaneously contain high levels of HSP70, this fact suggests that oocytes contain higher level of active HSF or, alternatively, different forms of CCAAT-binding transcription factor or HSF, or both (8).

With respect to this point, our experiments provide direct evidence for the presence of high levels of HSE-binding activity under nonshock conditions in cells that exhibit high levels of basal expression of two heat shock proteins, HSP86 and HSC73. Since our experiments involved only HSE, HSE-binding activity did not seem to require the binding of an auxiliary factor to a regulatory sequence distinct from HSE. In vivo, however, high basal transcription may depend on additional regulatory sequences and on other transcription factors. Indeed, the *Drosophila* HSP70 gene is not more highly expressed in EC cell lines than in fibroblasts and in differentiated 1009 cells (29). This gene does not contain a CCAAT box, in contrast to the rat HSC73 promoter, for example. This finding suggests that transcriptional activation in nonshocked EC cells requires additional factors such as CCAAT-binding transcription factor (as suggested for *Xenopus* HSP70 genes).

We pointed out the correlation between the presence of constitutive HSE-binding activity and high spontaneous HSP gene transcription in EC cells. Other authors had previously noted a correlation between the levels of E1A-like activity in EC cell line F9 and high spontaneous HSP expression (20, 26). It has been suggested that E1A acts by increasing the concentrations of cellular transcription factors (24, 49) or by modifying them (17, 18, 24, 42). It would therefore be interesting to determine whether the high constitutive level of HSE-binding activity in EC cells is due to E1A-like activity.

Though it is attractive to postulate that HSF acts in high constitutive expression of HSP86 and HSP73 in EC cells, one cannot exclude the involvement of factors completely distinct from HSF. In addition, we do not know whether the spontaneous HSE-binding activity that we detect in EC cells has any transcriptional activity. An HSE-binding activity, though not active for transcription by itself, may cooperate with other transcription factors to form an active transcription complex.

Inability of noninducible EC cells lines to induce HSE-binding activity upon heat shock. We demonstrated that HSE-binding activity is present in unstressed noninducible EC cell lines but that heat shock does not produce an increase in HSE-binding activity. On the contrary, heat-shocked cells contain much less binding activity than do nonshocked cells. These results warrant several remarks. (i) Noninducible EC cells are not deficient in HSE-binding activity, since such an activity exists at significant levels in control cells and at much lower but detectable levels in heat-shocked cells. (ii) The presence of a constitutive HSE-binding activity at 37°C does not by itself prevent an increase in HSE-binding activity upon a stress, since F9 cells contain a constitutive HSE-binding activity and yet are able to induce HSE-binding activity upon heat shock. (iii) The failure to increase the level of HSE-binding activity after heat shock correlates with a decrease of the constitutive binding form. This decrease could be due to inactivation by changes in postranslational modifications (as with the translation initiation factors eIF2 and eIF4. [14]) or to insolubilization (as with the c-*myc* proteins [15, 28]). We tried to determine whether HSE-binding activity was trapped by association with an inhibitor (such as NF-κB in pre-B cells [4]) by subjecting extracts from 1009 heat-shocked cells to gentle dissociating agents (deoxycholate alone or with formamide as described by Bauerle and Baltimore [4]), but these treatments did not reveal any masked HSE-binding activity (data not shown). (iv) It remains to be determined whether the constitutive and the heat-induced HSE-binding activities are distinct. We may be dealing with two different forms of HSE-binding activity, because although unstressed EC cells contain constitutive HSE-binding activity, they do not exhibit significant transcription rates from the strictly heat-inducible *Drosophila* HSP70 promoter (29). Finally, there are at least two explanations for the inability of noninducible cells to induce HSE-binding activity after a stress. Noninducible EC cells may be either completely deficient in heat-activable HSF species or deficient in the activation of HSF. During the differentiation process, the cells would synthesize the heat-activable form of HSE-binding activity or, alternatively, the mechanism of activation of HSF would become functional.

EC cell lines appear to provide a convenient system for investigation of the mechanisms of activation and inactivation of HSF in mouse cells as well as the involvement of HSF in constitutive HSP gene transcription. These cells may also provide a good model for study of the appearance of heat inducibility in early embryogenesis. Indeed, all noninducible EC cell lines tested behave identically with respect to the constitutive presence of HSE-binding activity and the absence of heat stimulation. Therefore, it may be that eight-cell embryos in which HSP synthesis cannot be induced by stress are also unable to produce high HSE-binding activity upon stress (29, 30). This ability would be acquired during the formation of blastocysts, since it appears during the differentiation of 1009 cells. In addition, embryos at preimplantation stages may spontaneously contain significant levels of HSE-binding activity, which could account for the high spontaneous expression of HSP86 and HSC73 at these stages.

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